

HUMAN COAGULATION FACTOR VIII

PURIFICATION, CHARACTERIZATION AND BIOLOGICAL INTERACTIONS

by

Karin Sewerin



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ABSTRACT

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Human plasma factor VIII has a molecular weight of 280 kDa and is composed of two peptide chains with a molecular weight of 200 and 80 kDa, respectively. The peptide chains are held together by one or several metal ion bridges. These metal ions are essential for the biological activity of factor VIII. Factor VIII is very susceptible to proteolytic degradation. Partially degraded but active forms of factor VIII with a molecular weight ranging from 280 to 170 kDa were isolated from commercial factor VIII concentrates.

The association between factor VIII and vWf in plasma constitutes an equilibrium process in which about 10–20% of factor VIII circulates in a free form. The binding site for vWf is located on the N-terminal part of the light chain of factor VIII. This bond dissociated on increasing the concentration of CaCl_2 , starting at 20 mM CaCl_2 . The binding is not mediated by metal ions, however.

Factor VIII is bound to certain phospholipid preparations or to the surface of released platelets. This binding induces a dissociation from of the vWf protein and the antigenic concentration of factor VIII was diminished by the binding to phospholipids. Factor VIII was activated by incubation with trace amounts of thrombin. The rate and degree of activation is dependent on the concentration of thrombin. Proteolytic cleavages in the factor VIII molecule are related to the increase in activity. The factor VIIIa formed is very unstable and the activity is subsequently decreased. The inactivation is probably due to a conformational change in the factor VIII molecule rather than proteolytical cleavages by thrombin.

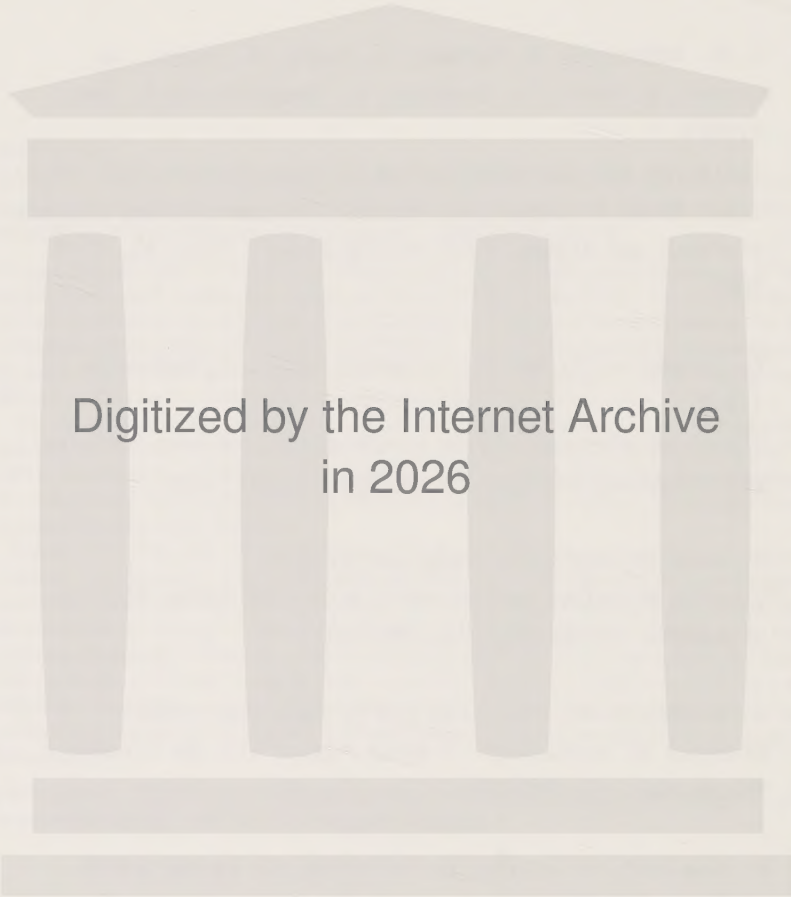
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PAPERS DISCUSSED

The present thesis is based on the following articles, which will be referred to in the text by the Roman numerals I to VI.

- I K. Sewerin* (1986).
Factor VIII-von Willebrand factor interactions in plasma and in purified state. J.Lab.Clin.Med., submitted for publication.
- II L.-O. Andersson, N. Forsman, K. Huang, K. Larsen, A. Lundin, B. Pavlu, H. Sandberg, K. Sewerin* and J. Smart (1986).
Isolation and characterization of human factor VIII. Molecular forms in commercial factor VIII concentrate, cryoprecipitate and plasma. Proc.Natl.Acad.Sci. (USA) 83, 2979-2983.
- III K. Brodén, J. E. Brown, C. Carton and L.-O. Andersson (1983).
Effect of phospholipid on factor VIII coagulant activity and coagulant antigen. Thromb.Res. 30, 651-660.
- IV K. Sewerin* and L.-O. Andersson (1983).
Binding of native and thrombin activated factor VIII to platelets. Thromb.Res. 31, 695-706.
- V K. Brodén, L.-O. Andersson and H. Sandberg (1980).
Kinetics of activation of human factor VIII by thrombin. Thromb.Res. 19, 299-307.
- VI K. Sewerin*, A. Lundin, E. Hellström, K. Larsen and H. Sandberg (1986).
Activation and inactivation of human purified factor VIII by thrombin. Biochemistry, submitted for publication.

* born Brodén



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Vad är sanning

'Då frågade Pilatus: Vad är sanning?'
och eko svarade - profeten teg.
Med gåtans lösning bakom slutna läppar
till underjorden Nazarenen steg.

Men gudskelov, att professorer finns,
för vilka sanningen är ganska klar!
De äro legio, ty de äro många,
som skänkt den tvivelsamme romarn svar.

Dock syns mig sällsamt, att det enda sanna
så underbart kan byta form och färg.
Det, som är sanning i Berlin och Jena,
är bara dåligt skämt i Heidelberg.

Det är, som hörde jag prins Hamlet gäcka
Polonius med molnens gyckelspel:
'Mig tycks det likna si så där en vessla
- det ser mig ut att vara en kamel!'

Gustav Fröding

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ABBREVIATIONS, DEFINITIONS

CPD	Citrate phosphate dextrose
DFP	Diisopropyl fluorophosphate
EDTA	Ethylenediaminetetra-acetate
EGTA	Ethylene glycol bis(β -aminoethylether)- N, N, N', N''-tetraacetic acid
Factor VIIIa	The activated form of factor VIII
HSA	Human serum albumin
BSA	Bovine serum albumin
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PL	Phospholipid
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
SDS	Sodium dodecyl sulphate
Tris	Tris-(hydroxymethyl)aminomethane
T _{1/2}	Half-life
Da	Dalton

NOMENCLATURE OF THE FACTOR VIII-VON WILLEBRAND COMPLEX

Factor VIII (VIII)

Biological function: - Procoagulant cofactor

Assay:

Functional (VIII:C) - Partial thromboplastin time
- Factor Xa formation

Immunological (VIII:Ag) - IRMA, ELISA, immunoblot
- Antibody neutralization

von Willebrand factor (vWf)

Biological function: - Platelet adhesion to the vessel wall
- Carrier for factor VIII in plasma

Assay:

Functional - Bleeding time, platelet adhesion,
ristocetin-induced platelet
agglutination

Immunological (vWf:Ag) - IRMA, ELISA, electroimmunoassay
- Immunoblot, multimer analysis

INTRODUCTION

Haemophilia A, historical background

The factor VIII protein has been the subject of intense research during the last three decades, but the earliest observation concerning haemophilia is actually very old. The Babylonian Talmud, probably from the third century AD, states: "It has been reported of four sisters of Sephors; the first one circumcised her son; and he died; the second and he died; the third and he died; the fourth came to Rabban Ben Gamakel, who said to her; abstain from circumcision for there are families whose blood is loose, while in others it coagulates" (1).

In the 12th century several men from an Arabian village were described as bleeding to death after trivial wounds (2).

Abnormal bleedings have been described through the centuries, one report was given in 1813 by Dr John Hay, USA, in "Account of a Remarkable Haemorrhagic Disposition Existing in Many Individuals in the Same Family" (3).

The most famous family affected with haemophilia was that of Queen Victoria of England. It is not known whether the defect originated from a mutation in the X chromosome of a germ cell of her father, or if this disorder was a result of a mutation in a gene in one of her own X chromosomes. However, she introduced the disorder to the European Royal families through three of her nine children. One of her sons, Leopold suffered from repeated episodes of bleeding and died from a bleed after a minor injury, at the age of 21. Two of Queen Victoria's daughters, Alice and Beatrice, definitely proved to be carriers and passed the mutation to their children. Alice was married to Louis IV, Grand Duke of Hesse. One son of their six children died at the age of three from haemophilic haemorrhage. Two of their daughters, Alexandra and Irene, were carriers. Alexandra was married to the last Russian Czar, Nicholas II. Their son, Czarevitzu Alexis, proved to be haemophilic. He was treated after a number of severe bleedings by a fanatic monk, Rasputin, who kept the child out of danger, by replacing the wild plays with prayers and meditation. The whole family was executed after having been

arrested in 1918. Beatrice, the second daughter of Queen Victoria who was a carrier of haemophilia, passed the trait to Spain through one of her daughters who married King Alfonso XII. Two of their sons, or perhaps three, died of haemorrhages (4).

Although there were several very old observations of this bleeding disorder (2) (5), which was first named haemorrhaphilia (love of bleeding) by Professor Schönlein (1793-1864), very little was understood about the function of blood coagulation until the 20th century. The first description of haemophilia as a protein disorder was given by Mr Ward in 1835, who reported that the blood of an individual with haemophilia-like disease did not coagulate at all. A few years later the first transfusion of blood to treat a haemorrhage was made by Dr Lane in London (6).

In the late nineteenth century fibrin was isolated by Hammarsten and at the same time thrombin was described as an enzyme for the conversion of an inactive precursor to fibrin strands. Morawitz postulated a "Theory of Blood Coagulation" in 1905, comprising two phases (7) (see fig. 1).

Activation of prothrombin to thrombin and conversion of fibrinogen to fibrin.

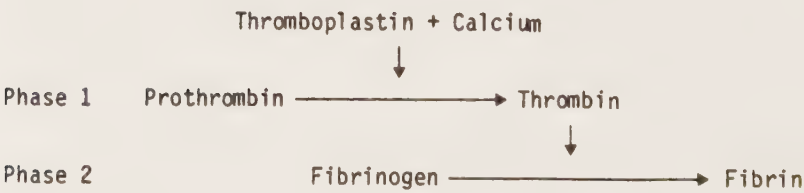


Fig. 1. "Classical coagulation theory", Morawitz in 1905.

His ideas about the coagulation system have been shown to be correct, although a number of proteins essential for the formation of the fibrin clot have been added.

Addis showed in 1911 that a plasma globulin could normalize the prolonged clotting time in plasma from haemophiliacs. This protein was partially purified and isolated in a crude form by Patek and Taylor in 1939 and was named antihaemophilia globulin. In 1947 Pavlovsky discovered that haemophilia was two distinguished disorders, haemophilia A and haemophilia B, due to the lack of two separate proteins (8). This phenomenon was clarified a few years later and the two forms of bleeding disorder were defined as: haemophilia A, due to the lack of antihaemophilic factor, now called factor VIII, and haemophilia B, expressed in patients deficient in a protein called the Christmas factor, or factor IX.

The term factor VIII refers to the deficient coagulant activity in classical haemophilia, a disease associated with bleedings into muscles and joints. The disorder is an X-linked recessive trait, which means that females carry the disposition without developing the disorder, whereas males with a defective gene in the X-chromosome have the disease in a more or less expressed form. The bleeding disorder was classified as severe if the factor VIII activity amounted to < 1%; moderate if it amounted to 1-4% and mild if it amounted to 5-25% of the activity in normal plasma (9).

It was found, however, that a factor VIII deficiency also occurs in another disorder: von Willebrand's disease. This disease is, in combination with the factor VIII deficiency, associated with prolonged bleeding times and defective platelet adhesion. The disorder is controlled by an autosomal gene, expressed by low levels of functional von Willebrand factor protein (vWf). The level of vWf in haemophiliacs is normal. It was now shown that factor VIII and vWf are two biochemically and functionally distinguished entities under separate genetic control. During the 1970s there was an intense discussion as to whether factor VIII and vWf were one molecule, two molecules or a protein complex (10).

Treatment of haemophilia A

In 1956, when Blombäck and Blombäck developed a method for purification of fibrinogen by a Cohn fractionation system, it was discovered that factor VIII was recovered almost quantitatively in a fraction called fraction 1-0 (11). This method for the preparation of factor VIII for therapeutic use in the treatment of haemophilia A and vWf-disease has been used in Sweden since 1956 (12). A series of purification methods was developed including the use of alcohol, cryoalcohol, or polyethyleneglycol precipitation steps, and today concentrates are purified up to 100-fold compared to plasma, with an activity of about 20-60 IU/ml. These modern fractionation and purification methods have made prophylactic treatment of haemophiliacs possible. Haemophilia A youngsters in Sweden are given 25 IU of factor VIII per kg every 3rd to 7th day from the age of five to about 18-20 years. This level is sufficient to prevent serious internal bleeds and allows the boys to have a nearly normal childhood. One severe haemophilia A patient is consuming about 100,000 IU per year, this can be expressed as a consumption of blood from 800 double plasmapheresis donations per year. For acute surgical treatment, large amounts of factor VIII are given up to a level of 30 to 70% of normal plasma during the operation and to 20-40% in the post-operative course. The $T_{1/2}$ of factor VIII from a commercial high purity concentrate is about 12 hours, which means that a patient can be given very large amounts of factor VIII during one period of treatment.

The massive treatment with plasma-derived material is connected with high risks of virus transmission, such as hepatitis B, hepatitis non A-non B or HIV infection. Another problem with the multiple transfusions of plasma is induction of antibodies against factor VIII, which develops in about 10% of the haemophiliacs. Biggs et al. (13) (14) have characterized these antibodies and found that there are two types of antibodies against the factor VIII protein. In one type, found in 'low responder patients', the inhibition of infused factor VIII is first very rapid but reaches a plateau when the free antibody is bound to factor VIII, in circulating antigen - antibody complexes. Additional administered factor VIII is not neutralized and the patient can be

treated with normalized procoagulant activity in plasma. In another type, characterized in 'high responder patients', the administered factor VIII is neutralized but no plateau is reached and additional factor VIII is inactivated. These patients are treated with large amounts of porcine factor VIII (15) or with immunosuppressive therapy in combination with factor VIII (16).

Basic mechanisms in blood coagulation

The functional interrelationship between factor VIII and von Willebrand factor is still not solved. Both proteins participate, although on distinct levels, in arresting bleeding when a blood vessel is injured. This mechanism is a complex series of important reactions in the control of blood loss.

Firstly, the characteristics of the surface of the injured vessel are different from those of an intact vessel, which change triggers the platelets to become sticky and to adhere to the surface structures. In the formation of a platelet plug in the primary stage of haemostasis, the von Willebrand factor plays an important role. Platelets from patients with von Willebrand's disease do not adhere normally to a damaged vessel.

Secondly, the platelet content is released, including ADP and vasoactive amines, which cause a contraction of the injured vessel, and procoagulant phospholipids (PF3) on the surface of the platelets are exposed.

Thirdly, the coagulation mechanism is triggered, involving contact activation on subendothelial structures, exposure of tissue factor in the damaged tissue and a series of enzymatic reactions resulting in the formation of an insoluble clot composed of cross-linked fibrin strands.

Finally, when the vessel wall is healed the fibrinolytic system is activated to degrade the fibrin clot.

Although the fundamental principles of blood coagulation have been known for about 80 years, the major advances in our understanding of blood coagulation have been made during the last two decades. A large number of proteins are involved in the coagulation process, most of

which have now been well characterized, both functionally and biologically.

The formation of fibrin is the result of a series of stepwise activations of proenzymes present in plasma to enzymes. This reaction scheme has been referred to as a waterfall (17), or a cascade (18), as the reactions show an amplification in that a few molecules in the first step activate some hundred proenzyme molecules in the second, which in turn can activate thousands of molecules in the next, and so on (fig. 2). Several proteolytic cleavages of well-defined peptide bonds are involved in this activation process (19).

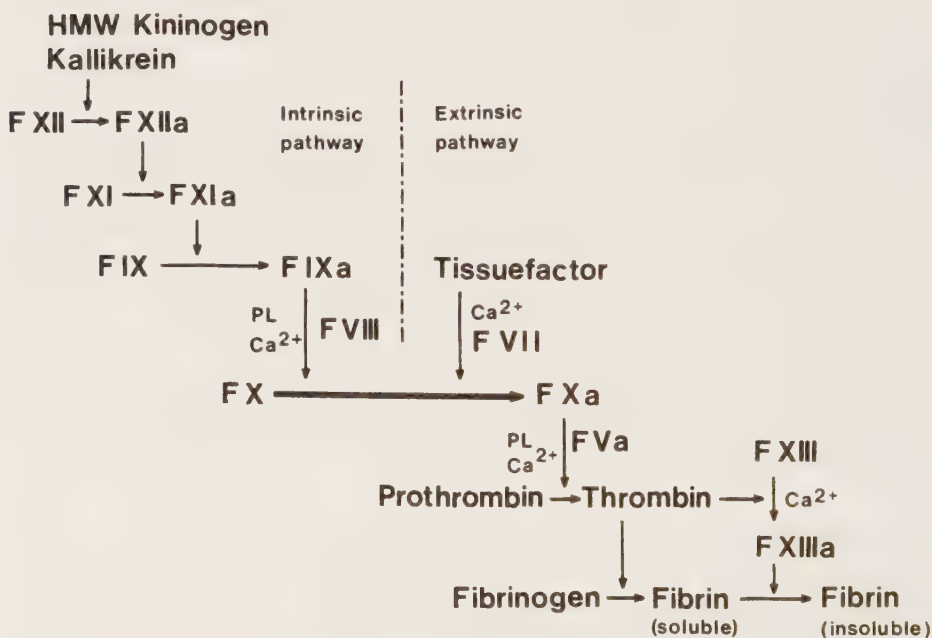


Fig. 2. Coagulation cascade.

The coagulation process can be initiated in at least two different ways: by the intrinsic and by the extrinsic pathway. The first activation step in the intrinsic pathway is the conversion of the inactive precursor factor XII to an active enzyme factor XIIa. This activation takes place on a negatively charged surface, in vitro, on the wall of a

glass tube, for example. The conformation of factor XII is changed, making it more sensitive to proteolytic cleavage. Kallikrein, which is also adsorbed to these surfaces, converts the one-chain form of factor XII by several cleavages to an active two-chain form, also called γ -factor XIIa or factor XIIa⁺. The thus formed enzyme activates the next precursor in the cascade, factor XI, to an active enzyme, factor XIa. This activation involves an internal cleavage of the protein. The next precursor to be activated is factor IX, which is activated by factor XIa, including cleavage of the single-chain polypeptide to an inactive intermediate form composed of two polypeptide chains. An active enzyme is then formed by a second cleavage in one of the chains. Factor IXa is the serine protease which, in the presence of a cofactor VIIa, phospholipid and CaCl_2 , activates the precursor factor X to an active enzyme, factor Xa. Factor IXa and factor X are bound to the phospholipid, in vivo to the surface of the platelets, by Ca^{2+} bridges, and an activated form of factor X is split off from the complex. The role of factor VIII in this complex, also called "the tenase complex" will be discussed later.

An alternative route for the activation of factor X to factor Xa is by the extrinsic pathway, in which the tissue factor and factor VII are the two active components. The tissue factor is a lipoprotein that can be isolated from many different tissues. Factor VII is, like many other coagulant proteins, a precursor that can be activated by factor XIIa, by factor IXa, by factor Xa or by thrombin. This activation includes an internal cleavage of the single-chain polypeptide chain with formation of an active enzyme. Factor VIIa forms a complex with the tissue factor and Ca^{2+} ions and activates factor X to factor Xa in the same way as the tenase complex does. The extrinsic pathway of factor X activation is much faster than the intrinsic one, but both are essential for normal coagulation (20).

The next step in the coagulation cascade is the activation of prothrombin (factor II) to thrombin (factor IIa) by the "prothrombinase complex", common to both the intrinsic and the extrinsic pathways. The activation complex involves the enzyme factor Xa, phospholipid, Ca^{2+} and

factor Va. This activation is much like the conversion of factor X into an active enzyme by 'the tenase complex'. Factor Xa is bound to specific sites, phospholipid receptors on the surface of the activated platelets, the binding being mediated by Ca^{2+} ions. (21). The activity of factor Xa is increased more than 50,000-fold by the binding to the platelets. Factor V participates in the complex after activation by trace amounts of thrombin (22). The single-chain protein with a MW of 330 kDa is cleaved and two polypeptides (94 and 44 kDa), held together by internal Ca^{2+} bridges, form the active protein (23). Factor Va also binds to the negatively charged phospholipids on the platelet (24) (25).

Fibrin, the final product in the coagulation process, is formed by a stepwise conversion of fibrinogen, involving proteolytic cleavage, polymerization and stabilization. Fibrinogen is a plasma protein consisting of two identical parts, each of them containing three peptide chains called A α , B β , γ . In the first step of the activation two peptides are split off from fibrinogen, A from the A α chain and B from the B β chain. This leads to a conformational change in the molecule and to polymerization of the fibrin monomers and a soluble fibrin clot is formed. To stabilize this aggregate, covalent bonds between the fibrin monomers are formed. Factor XIIIa, activated from its precursor factor XIII by thrombin, catalyzes this process, resulting in a cross-linked insoluble fibrin clot that is considerably more stable against proteolytic degradation.

Several positive and negative feed-back reactions are involved in the coagulation process. These regulatory mechanisms start with the contact activation when kallikrein accelerates the process. The concentration of thrombin is very important for the activation and inactivation of the cofactors factor VIII and factor V. The coagulation is a very unstable process and a regulatory system is necessary to control these activities. A series of serine protease inhibitors, such as α_2 macroglobulin and C_1 esterase, are present in plasma during the coagulation. Antithrombin III forms a complex with thrombin, factor IXa, factor X, factor XIa and factor XIIa, resulting in an irreversible inhibition of

their proteolytic activity. Heparin increases the rate of binding between antithrombin and the enzyme. Protein C is a potent inhibitor of factor VIII and factor V.

Many of the proteins participating in the coagulation process - prothrombin, factor X, factor VII and factor IX - require vitamin K for the synthesis of a biologically active protein (19) (26) (27). In the presence of vitamin K a number of glutamic acid residues in the N-terminal part of the protein are carboxylated, forming γ -carboxyglutamic acid (26) (28). The carboxylation is essential for the binding of Ca^{2+} to the protein, which then implements the binding to negatively charged phospholipid membranes (25) (29).

AIMS OF THE PRESENT INVESTIGATION

The purification and characterization of the factor VIII protein have been the subject of intense research during the past 30 years. The lability of the protein and its low concentration in blood have made this work very difficult. The aims of the present investigation were to:

- Purify and characterize human coagulation factor VIII.
- Study the interaction between factor VIII and von Willebrand factor.
- Clarify the effect of phospholipids on factor VIII.
- Elucidate the mechanisms for the activation of factor VIII by thrombin and to characterize the factor VIIIa formed.

The von Willebrand factor protein

In the blood of normal individuals the factor VIII protein circulates mainly in association with the von Willebrand factor protein (vWf) in a macromolecular complex. The vWf protein is composed of a series of high molecular weight oligomers, each of which is built up from a number of subunits with a MW of 220 kDa. The subunits form either a dimer (30) or a tetramer (31), held together by disulphide bridges. In the blood circulation these protomers form multiples with a MW of up to 20×10^6 Da. The bonds holding the large multiples together are more accessible to reducing agents than those holding the dimers or tetramers together (30).

The biological function of the vWf protein is to promote adhesion of platelets to the subendothelium (32). The specific test for vWf activity is correction of the bleeding time defect in patients with vWf disease. For practical reasons, a variety of in vitro techniques have been devised for assessing activities reflecting the vWf activity, using a washed platelet system and the antibiotic, ristocetin (33). Quantitative and qualitative methods for an immunological determination of vWf protein using antibodies to vWf can also be used (34). The concentration of vWf in plasma is 5-10 $\mu\text{g/ml}$, which is defined as 1 U/ml normal plasma.

The factor VIII - vWf complex

The vWf also functions as a carrier for the factor VIII protein (35). It is generally accepted that the two protein entities circulate as a non-covalently linked protein complex (31) (36), although the exact relationship between the two proteins still remains unknown. The association between factor VIII and vWf in plasma is thought to be an equilibrium process (I) (37), in which about 10-20% of the factor VIII protein is present in a free non-complexed form. This was demonstrated when factor VIII from fresh plasma, collected in the presence of pro-

tease inhibitors, was sedimented by ultracentrifugation in a sucrose gradient (1). In conformity with these results, Weinstein et al. suggested some years ago that one part of the factor VIII was present in a low molecular weight form in plasma. Factor VIII related activity was found in the cryosupernatant, measured by antibodies to the factor VIII protein, when fresh plasma was precipitated. The factor VIII that was bound to vWf protein was found in the cryoprecipitate. This was also demonstrated by gel filtration experiments (38).

The factor VIII protein can be dissociated from the vWf protein in high ionic strength buffers or in a system containing a high concentration of CaCl_2 (36) (39). Owen and Wagner reported that Ca^{2+} ions are specific and more potent as dissociating agents than any other mono- or divalent ion tested (40). A dissociation between factor VIII and vWf protein starts to occur in the presence of 10-20 mM CaCl_2 , as demonstrated by sucrose density gradient centrifugation of fresh plasma in the presence of protease inhibitors (1) or by gel filtration in a buffer containing CaCl_2 (41). A complete dissociation between factor VIII and vWf is noted at about 0.2 M CaCl_2 . This has been demonstrated by gel filtration in a buffer containing 0.25 M CaCl_2 , (36) (39), by ultracentrifugation experiments (1) (41) (42), and by immunoabsorption chromatography with antibodies to vWf, followed by dissociation of factor VIII with CaCl_2 (41) (42). (See below.)

The factor VIII protein

The concentration of factor VIII in plasma is about 150 ng/ml, corresponding to a molar concentration of 0.5 nM. This very low concentration of factor VIII makes it very difficult to study when complexed with the vWf protein. The biological activity of factor VIII is defined as the procoagulant activity in normal plasma, expressed as 1 IU/ml (43). This functional activity can be estimated by a clotting assay, using a factor VIII deficiency plasma as substrate (44), or by a chromogenic substrate assay (45). The factor VIII protein can also be determined by estimating the factor VIII:Ag concentration, with poly- or monoclonal antibodies to factor VIII by radioimmunoassay (46) (47), or by enzyme immunoassay (48).

There is no general agreement about the size of the native factor VIII molecule. A sedimentation coefficient of about 8 S was found when factor VIII was dissociated from vWf in plasma by means of sucrose density gradient centrifugation in highly concentrated CaCl_2 in the presence of protease inhibitors throughout the experiment (I) (42). This value of the sedimentation coefficient corresponds to a protein with a MW of about 275 kDa, assuming normal globulin shape and hydration. The same result was obtained from calculations of the estimated value of Stoke's radius determined by gel filtration experiments (49).

In 1978 Holmberg and Ljung described a method for the purification of factor VIII by immunoabsorption chromatography (50). The factor VIII-vWf complex was adsorbed to antibodies to vWf and factor VIII was eluted by dissociation from vWf with a highly concentrated CaCl_2 . This method has been widely used since then, utilizing monoclonal or polyclonal antibodies against vWf as well as against factor VIII. Several groups have reported characteristics of highly purified factor VIII produced by this method in combination with other techniques (see below).

In our laboratory factor VIII was isolated from human plasma collected in the presence of protease inhibitors by gel filtration or by immunoabsorption chromatography (II). The factor VIII material was analysed by SDS-PAGE and immunodetection techniques using antibodies against factor VIII. These data suggest that factor VIII is a two-chain protein with a MW of 280 kDa, consisting of one heavy chain of 200 kDa and one light chain of 80 kDa. Hoyer and Trabold reported a MW of 285 kDa for native factor VIII purified from fresh plasma, in the presence of protease inhibitors, using the immunoabsorbent technique. The estimation of the molecular weight was made by gel filtration on Sephadex 100 (49). In contradiction to these findings, Rotblat et al. have described a single-chain form of factor VIII with a MW of ~ 365 kDa (51). This factor VIII material was purified from fresh plasma by cryoprecipitation and immunoabsorption chromatography. The material was characterized by SDS-PAGE and immunodetection methods using antibodies against factor VIII.

Highly purified factor VIII

Human factor VIII has been isolated and highly purified from commercial factor VIII-vWf concentrates, by immunoabsorption chromatography using antibodies to vWf, followed by HPLC ion exchange chromatography on Mono Q gel (II) or by hydrophobic interactions on aminohexyl Sepharose (52) (53). This material was purified up to 480,000-fold compared to plasma and with a specific activity of up to 6700 IU factor VIII/mg protein (II). Rotblat et al. have purified human factor VIII to about the same degree of purity from cryoprecipitate by aluminium hydroxide adsorption and polyelectrolyte purification, followed by immunoabsorption chromatography on antibodies against both vWf and factor VIII (51).

The highly purified factor VIII, from all preparations, showed a molecular weight heterogeneity, which indicates degradation during processing. A series of protein components with MW's ranging from 80 to 200 kDa was demonstrated by SDS-PAGE analysis under reducing conditions. Most of these were correlated with the factor VIII protein demonstrated by the immunoblot technique using monoclonal antibodies against factor VIII.

The heavy chain of 200 kDa, present in the factor VIII material isolated from fresh plasma, was degraded into peptide chains of 90 to 180 kDa. These fragments were shown by N-terminal amino acid analysis to be derived from the C-terminal part of the heavy chain. The light chain with a MW of 80 kDa was not changed during the purification. This peptide chain formed a complex with any of the partially degraded forms of the heavy chain. The two chains are held together by metal ion bridges (see below). All of these more or less fragmented forms of factor VIII possess factor VIII activity, the smallest active form of factor VIII being composed of one 90 kDa and one 80 kDa peptide chain (II). The 170 kDa form of factor VIII was shown to have the same *in vivo* survival in haemophilic dogs, about 9 hours, as the (280-185) kDa form. Association between the 170 kDa form of human factor VIII and the canine vWf was demonstrated by gel filtration experiments (54). The highly purified

form of factor VIII did not sediment as fast as the plasma form in a sucrose gradient, but a sedimentation coefficient of 7.2 S was noted. The difference is due to the lower average molecular weight in the purified state of factor VIII (I).

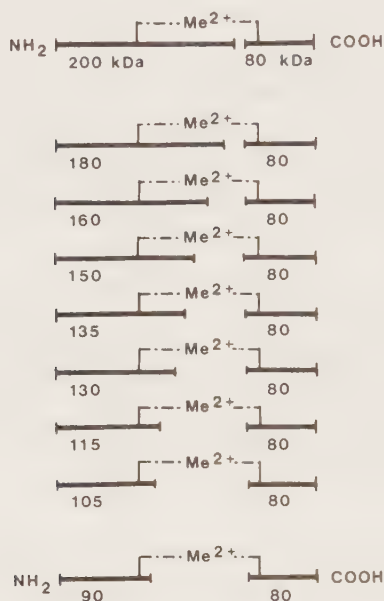


Fig. 3.

A model of the presumed form of plasma factor VIII with a MW of 280 kDa and the various fragmented and active forms of factor VIII.

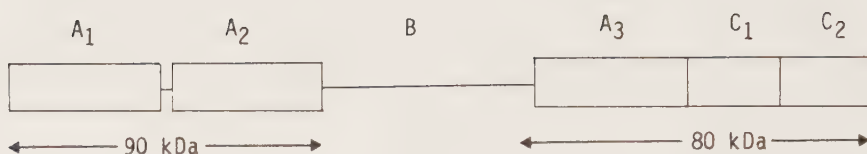
Factor VIII isolated from other species showed a high degree of homology with human factor VIII. Vehar and Davie purified factor VIII about 300,000-fold from bovine plasma, using conventional protein purification methods, including factor X-Sepharose column chromatography (55). This material had a MW of 250-300 kDa. The bovine factor VIII was shown, by SDS-PAGE analysis with or without reduction, to contain three polypeptide chains with MW's of 93 kDa, 88 kDa and 85 kDa. Porcine fac-

tor VIII was isolated by several purification steps including two immunoadsorption chromatography steps, using antibodies to both vWf and factor VIII. This preparation of factor VIII had about the same degree of purity as the bovine preparation. Porcine factor VIII was shown by SDS-PAGE analysis to contain subunits of 160 kDa, 130 kDa, 82 kDa and 76 kDa (56) (57).

The structure of factor VIII

cDNA clones that encode the total factor VIII molecule have recently been isolated by two independent groups (58) (59). Their data show that factor VIII is synthesized in a single-chain form, composed of 2332 amino acids with a theoretical molecular weight of 265 kDa (60). It is not clear, however, whether this form of factor VIII is a precursor form that has to be cleaved after biosynthesis to form a biologically active form or if this is the native form of factor VIII.

The amino acid composition of factor VIII shows that there are certain distinct domains with striking sequence homology. Fig. 4 shows a model of the single-chain form of factor VIII. Three domains, 'A', containing 330 amino acids each, show about 30% internal homology. Two of these are located in the N-terminal part of the molecule, separated from the third 'A' domain by a unique 'B' domain, which consists of 980 amino acid residues. The third 'A' domain is linked to one of two 'C' domains, which consist of 150 amino acids and have an internal sequence homology of about 35%. This region is located in the C-terminal part of the molecule (60). The 'A' domains do not only show internal homology, but also a 30% sequence homology with two copper-containing plasma proteins, ceruloplasmin (61) (62) and factor V (63) (64) (65).



A₁, A₂, A₃-30% pairwise aminoacid homology

C₁, C₂ -37% aminoacid homology

A₁, A₂, A₃-30% pairwise aminoacid homology
with ceruloplasmin

Fig. 4. Single-chain form of factor VIII.

When compared with the polypeptide structure of factor VIII purified from plasma or factor VIII-vWf concentrates, it can be seen that the two N-terminal 'A' domains represent the 90 kDa peptide chain of the heavy chain (Fig. 5). The combination of the third 'A' domain and the two 'C' domains forms the 80 kDa peptide chain (60). This indicates that a cleavage rapidly occurs between the 'B' domain and the A₃-C₁-C₂ region (the 80 kDa peptide chain) producing the two-chain form of factor VIII.

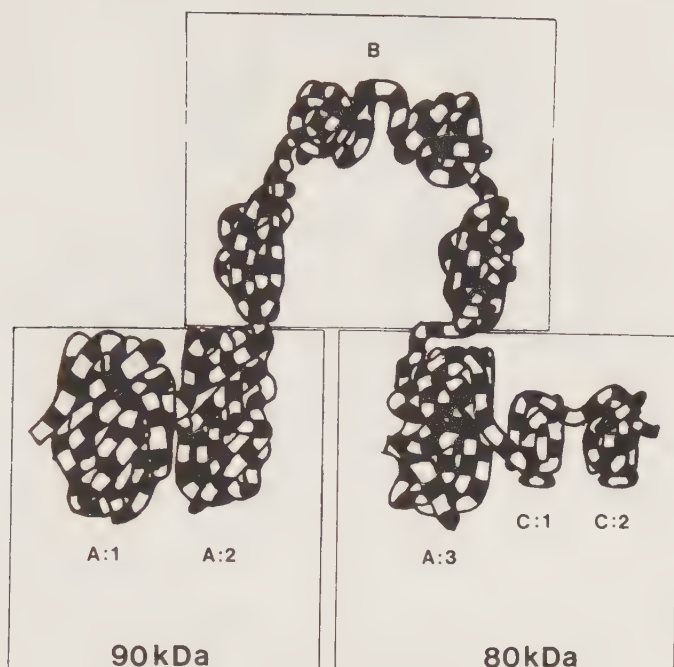


Fig. 5. Model of the structure of factor VIII.

The intermediate chain, the 'B' domain, between the two functional regions of the molecule, is very sensitive to proteolytic degradation and is readily cleaved so as to produce the various fragmented forms of factor VIII present in factor VIII material purified from plasma concentrates (II). The function of this domain is still not known, but it has been suggested that a major portion of glycosylation sites on the factor VIII molecule are located within this area. Twenty-five possible N-glycosylation sites can be predicted from the amino acid composition of factor VIII, 19 of them being found in the 'B' domain (60). Fay et al. reported that the carbohydrates represent about 5% of the total molecular mass of factor VIII (66). The role of the carbohydrate resi-

dues on factor VIII in the procoagulant activity is still not clear, but Fukui et al. suggested from a study on the factor VIII-vWf complex that mannose and galactose are important for the function of factor VIII (67). However, Sodetz et al. reported from findings on the factor VIIIvWf, that the factor VIII activity was not influenced by removal of the carbohydrate residues using neuroamidase or β -galactosidase (68).

Factor VIII: A metal ion containing protein

Anticoagulants, like citrate or oxalate, are used to prevent blood from clotting during collection and storage. The effect of these chelating agents on the stability of factor VIII has been discussed for many years. In 1955 Spaet et al. showed that a higher concentration of chelating agents decreased the stability of factor VIII (69).

An addition of Ca^{2+} ions or other divalent ions can stabilize the factor VIII activity in plasma collected with citrate as an anticoagulant (70). Mikaelsson et al. showed that the loss of factor VIII activity was diminished when heparin was used as an anticoagulant or if CaCl_2 was added to heparinized CPD plasma (71). The results of their study suggested that factor VIII-vWf is a Ca^{2+} -containing protein complex and that the presence of ionized Ca^{2+} protect the factor VIII activity more effectively than any other mono - or divalent ion tested.

The heavy and the light chains of factor VIII are held together by one or several metal ion bridges (Fig. 6) (II). These metal ions are essential to the biological activity of factor VIII. Removal of the metal ions results in a total loss of factor VIII activity. Separation of the chains was recently demonstrated by gel filtration of highly purified human factor VIII after treatment with EDTA (II). No activity was found but the distribution of the peptide chains was demonstrated by SDS-PAGE analysis of the fractions.

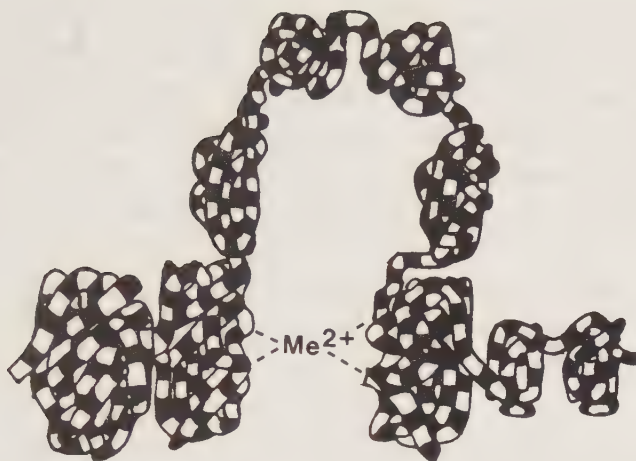


Fig. 6. Model of the structure of factor VIII with the presumed metal ion bridge in factor VIII.

This is in analogy with factor Va, which is known to contain a Me^{2+} bridge holding the heavy and light chains together. The presence of Ca^{2+} ions is also essential for the procoagulant activity of factor Va (72) (73). The sequence homology of factor VIII with ceruloplasmin and factor V, both of them Me^{2+} -containing plasma proteins, also implies that factor VIII has similar metal ion binding characteristics (60).

The binding of factor VIII to vWf

As discussed above, the binding between factor VIII and vWf was demonstrated to be a non-covalently linked association. Mikaelsson et al. showed that calcium ions are present in the factor VIII-vWf complex (71). Removal of these Ca^{2+} ions by chelating agents such as EDTA and EGTA resulted in a total loss of the factor VIII activity and also in dissociation of vWf and factor VIII:Ag measured with the aid of human antibodies to factor VIII (71) (74). However, evidence for separation of the entire factor VIII molecule from the vWf protein by treatment with EDTA was not demonstrated.

In our laboratory it has been shown by gel filtration of highly purified ^{125}I -factor VIII after incubation with vWf and treatment with

EDTA, that the binding between factor VIII and vWf is not dependent on Ca^{2+} ions. One part of the radioactivity, representing factor VIII material, was eluted in the void volume bound to the vWf protein (I). This was further demonstrated by insolubilized vWf to which factor VIII was bound (I) or by immunoadsorption chromatography (75), followed by dissociation of the chains with EDTA or EGTA. These results also showed that the binding site for vWf is located on the light chain of factor VIII. The same conclusion was reported by Hamer et al. from a study using monoclonal antibodies against the locus on the heavy and light chains, respectively (76).

THE PHYSIOLOGICAL ROLE OF FACTOR VIII (Papers III and IV)

The tenase complex

Factor X has a central position in the blood coagulation cascade. The activation of factor X by the intrinsic pathway involves the conversion of the proenzyme into an active enzyme. This activation of factor X is the result of a specific cleavage of a peptide bond, mediated by the serine protease factor IXa. Factor VIII participates in this process as a cofactor, part of the factor X activator, 'the tenase complex', in combination with factor IXa, Ca^{2+} , and phospholipid (77) (78) (79).

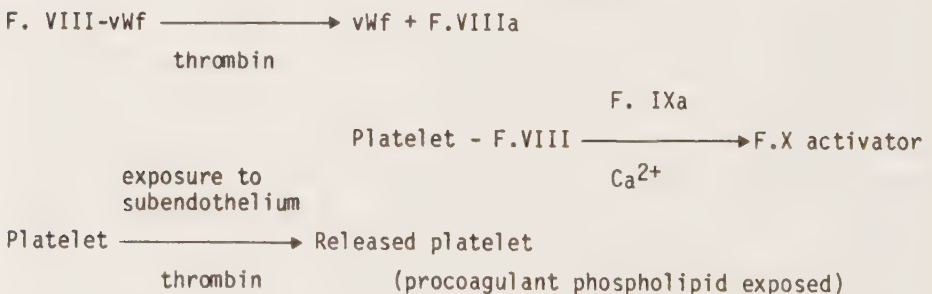


Fig. 7. Formation of the factor X-activator

Many groups have studied the kinetics of the activation of factor X to factor Xa in the presence of factor IXa, factor VIIa, phospholipid and Ca^{2+} , but the exact role of factor VIII in this reaction still remains unknown. For optimal cofactor activity, conversion of factor VIII into an activated form, factor VIIa, is required (80) (81) (82). These mechanisms will be discussed later.

Factor IXa is capable of activating factor X by itself, but under these conditions there is a lag period before the rate of activation is linear (78) (83). The addition of phospholipid to this system does increase the rate of the reaction, although the catalytic efficiency remains extremely low (84). The binding of factor X to phospholipid is mediated by Ca^{2+} ions, forming a factor X-PL- Ca^{2+} complex that is more efficiently activated by factor IXa than is free factor X. An excess of factor X makes the phospholipid the rate limiting component in this system.

Factor IXa is bound to phospholipids as well, the binding being mediated by Ca^{2+} bridges between the γ -carboxyglutamic acid residues in the factor IXa protein and the negatively charged phospholipids (85).

When factor VIIa was present in the system, the activation rate was increased a thousand fold and the reaction was stimulated (84) (86) (87) (88). Mertens et al. suggested that factor VIIa is bound to phospholipid and to factor IXa and that this is the functional factor Xa-activating enzyme (88). This suggestion was based on data indicating that only the phospholipid-bound fraction of factor IXa is bound to factor VIIa and that the contribution of factor VIIa to the formation of factor Xa is related to the concentration of factor IXa. Hougie et al. showed that factor IXa, factor VIII and phospholipid were eluted together as a complex when plasma was gel filtered in the presence of Ca^{2+} ions (89). Factor IXa was eluted separately if Ca^{2+} was omitted, indicating that factor IXa is bound to phospholipid via Ca^{2+} ions, whereas factor VIII is bound via a specific binding site (89) (90).

Binding of factor VIII to phospholipids and to platelets

The activation of factor X has much in common with the activation of prothrombin to thrombin by factor Xa in the presence of factor Va, phospholipid and Ca^{2+} ions. In the prothrombinase complex factor Xa and prothrombin bind to the phospholipid surface via calcium bridges between the γ -carboxyglutamic acids present in these proteins and the polar head groups of the phospholipid (91). In the binding of factor Va to the phospholipid membrane, hydrophobic interactions play an important role (92). The effect of phospholipids present during the activation of factor X to factor Xa has been discussed above. The binding of factors IXa and X to the phospholipid membrane is also mediated via Ca^{2+} bridges and it is likely that factor VIIId is bound to the phospholipid surfaces in the same way as factor Va.

The role of phospholipids in the activity and stability of factor VIII has been discussed by many groups. Barrowcliffe et al. observed that factor VIII was bound to phospholipid membranes or to platelets and that this binding protects the factor VIII molecule against antibodies to factor VIII (93). This was shown by a decreased measurable level of factor VIII:Ag after the addition of phospholipids or platelets to a factor VIII preparation (III) (93) (94). In vivo, this mechanism was used for the treatment of haemophiliacs with antibodies to factor VIII. The patients were treated with platelets that blocked the antigenic sites of the infused factor VIII (95).

The composition of the phospholipids is important for the binding of factor VIII. It has been demonstrated in our laboratory that phospholipid vesicles composed of PC and PE are not as effective in stabilizing the factor VIII activity as vesicles composed of equal amounts of PC and PS or PS and PE (III). PS was shown by Yoshioka et al. to bind to factor VIII to a higher extent than the other phospholipids tested, indicating that negatively charged phospholipid residues are needed in the interaction with factor VIII (96).

A dissociation between factor VIII and vWf occurs at a high concentration of phospholipid (III) (97). Andersson et al. showed that this dissociation is due to binding between factor VIII and the phospholipid (98). Factor VIII was bound to phospholipid vesicles consisting of equal amounts of PS and PE and was then separated from the vWf protein by ultracentrifugation. This was also demonstrated by gel filtration of factor VIII-vWf complex after incubation with phospholipid vesicles. When gel filtered on a Sepharose 2B column, the factor VIII activity was eluted in the void volume, bound to the vesicles (III). Comparable results were obtained when released platelets were used as the source of phospholipid (IV). The binding to platelets was more expressed when thrombin-activated factor VIII was used.

The binding to phospholipids does not only protect factor VIII against antibodies but also stabilizes the procoagulant activity (III). An increase in factor VIII activity was seen upon incubation of thrombin-activated factor VIII-vWf with a crude phospholipid concentrate or with phospholipid vesicles. This phenomenon might also be the effect of a stabilization of the activated factor VIII. (This will be discussed later.) Factor VIII bound to phospholipid was also found to be more extensively activated by thrombin.

Although a specific binding site for vWf has been demonstrated on human platelets (99) with a K_d of $4-5 \times 10^{-10}$ M (100), in the presence of ristocetin, there has been disagreement in the reports concerning the presence of a binding site for the factor VIII protein on the surface of the platelets. Raccuglia et al. and Karparkin et al. presented results indicating the presence of factor VIII on washed human platelets using coagulation methods for the detection of factor VIII (101) (102). However, Flaum et al. did not find any factor VIII material in washed human platelet preparations utilizing a human antibody against factor VIII (103). This can be explained by the use of an EDTA-containing buffer, which separates the heavy and light chains of factor VIII. If the antibody used was directed against the non-binding part of factor VIII, this would have been dissociated and not recognized on the platelets. Thrombin-activated factor VIII was shown by albumin density gradient centrifugation to bind to released platelets and to dissociate

from the vWf protein (IV). This indicates that there are specific binding sites for factor VIII on the surface of the platelets and that the factor VIII material found there is not bound to the vWf protein.

REGULATORY EFFECTS ON THE FACTOR VIII ACTIVITY (Papers V and VI)

Factor VIII is present in plasma in a very low concentration compared to the other coagulation proteins with which it interacts. A variation in the concentration of factor VIII may not play a critical role but the accessibility of factor VIII as an active cofactor in the activation of factor X is of great importance. The regulatory effect of several proteolytic enzymes, resulting in both positive and negative feedback on factor VIII activity, also have a considerable effect on the activation of factor X and, finally, on the generation of fibrin.

In the in vitro activation of factor X by factor IXa in the presence of factor VIII, there is a lag phase before the rate of activation becomes linear, as discussed above. This lag phase was reduced when factor Xa or thrombin was present, and it was completely eliminated when factor VIII was preincubated with factor IX (104).

Activation of factor VIII by thrombin

Trace amounts of thrombin enhance factor VIII activity, an initial increase in the activity being followed by a subsequent decrease and loss of activity (V). The exact mechanism of these reactions still remains unknown, but limited proteolytic cleavages in the factor VIII molecule have been shown to correlate with the activation process (VI).

Inhibition of the thrombin activity prior to incubation resulted in preventing the expected increase in factor VIII activity. Rick and Hoyer showed that DFP, a potent protease inhibitor that reacts with a serine residue in the active site of thrombin, inhibited the activation of factor VIII by thrombin (105). Hirudin is a specific thrombin inhibitor that was also shown to prevent the activation of factor VIII. This indicates that the formation of a more active form of factor VIII by

incubation with thrombin is due to the protolytic cleavages of factor VIII by thrombin.

Highly purified factor VIII was incubated with thrombin and samples from the incubation mixture were analysed by SDS-PAGE. A proteolytic cleavage of factor VIII by thrombin has been shown by several investigators to be related to an increase in the factor VIII activity (VI) (106). The heavy chain of factor VIII with a MW ranging from 105 to 200 kDa was degraded into a 90 kDa peptide chain, this chain was then subsequently cleaved to one 52 kDa and one 43 kDa peptide chain. A further cleavage of the 52 kDa peptide chain was seen upon prolonged incubation with thrombin (51) (107). The 90 kDa peptide chain and the 52 kDa fragment of this chain represent the N-terminal part of the heavy chain. The light chain of 80 kDa was also degraded with the formation of a 70 kDa peptide chain. The cleavage products of factor VIII formed by incubation with thrombin are shown in fig. 8. In the system tested, the degree of activation measured represents a balance between activation and inactivation of factor VIII in addition to the amount of factor VIII that is not activated. This complexity makes it difficult to distinguish between an activated and an inactivated form of factor VIII.

Rotblat et al. suggested that the 90 and 80 kDa peptide chains form the activated form of factor VIII (51). However, Andersson et al. showed that this form was activated by thrombin to about the same degree as the full-length form (II). Fulcher et al. suggested that the formation of the 90 kDa peptide chain is responsible, at least in part, for the activation of factor VIII (106). A further degradation of the 90 kDa peptide chain to 52 and 43 kDa peptide chains, in combination with the 70 kDa peptide chain, was suggested by Eaton et al. to be the fully activated form of factor VIII (108). We showed that one activated form of factor VIII was eluted when factor VIII bound to vWf-Sepharose, was treated with thrombin. This material was mainly composed of the 90 and 70 kDa peptide chains at the time of maximal activation, but the 52 and 43 kDa peptide chains were also present and increased in amount upon prolonged incubation (VI). This indicates that one activated form of factor VIII is composed of the 90 kDa peptide chain in combination with the 70 kDa peptide chain. However, it cannot be ruled out that more than one activated form of factor VIII exists.

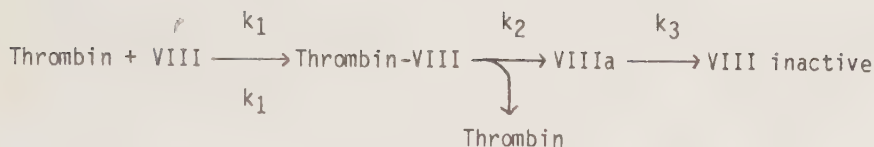
Many attempts have been made to stabilize the factor VIIIa activity and to characterize this form of factor VIII. Thrombin inhibitors like DFP, PMSF, hirudin etc. were added to the incubation mixture to prevent further degradation when a rise in the activity was seen (VI) (105) (109) (110). The factor VIII activity formed during the incubation was not preserved, despite complete inhibition of the thrombin activity. Removal of the thrombin after activation by immobilized thrombin did not prevent the loss of activity, although the peptide composition formed was stabilized. These findings indicate that the inactivation of factor VIII is due to an intrinsic instability in the factor VIIIa molecule rather than further proteolytic cleavage by thrombin. The rate of inactivation was diminished by lowering the temperature during the incubation (110).

The effect of Ca^{2+} ions on the activation and inactivation has also been studied by several groups (VI) (108) (110) (111). The presence of a high concentration of CaCl_2 during the incubation with thrombin was shown to partly stabilize the activated form of factor VIII, but the degree of activation was also limited in this system (110). On the other hand, Eaton et al. showed that the absence of additional CaCl_2 during incubation with thrombin was found to stabilize the factor VIII activity produced (108).

The factor VIII activity produced by incubation with thrombin was shown to be at least partly stabilized by adding albumin (VI). This supports the opinion that the degradation is due to a conformational change in the molecule.

Lollar et al. reported that factor IXa, in combination with phospholipid, preserves factor VIIIa activity (112). This suggests that factor VIIIa in 'the tenase complex' is stable, which is important for its biological function in the coagulation system.

Kinetic studies on the activation of factor VIII by thrombin indicate a first order reaction, which was demonstrated with both purified porcine factor VIII (113) and human factor VIII-vWf (V). The conversion of factor VIII to factor VIIIa by thrombin obeys Michaelis Menten kinetics, suggesting the following mode for the reaction:



This model includes the formation of a complex between factor VIII and thrombin. A proteolytic cleavage of factor VIII by thrombin results in the formation of activated factor VIII (VIIIa). The activity produced is subsequently abolished by a spontaneous first order reaction. An apparent K_m for the activation of factor VIII-vWf was estimated to be $4.3 \times 10^{-9} \text{ M (V)}$.

The formation of a complex of factor VIII-vWf and thrombin was demonstrated by gel filtration (V) and ultracentrifugation (110). This binding of thrombin is specific for factor VIII-vWf and DFP-treated thrombin was demonstrated to bind as well as active thrombin (110). Hau et al. recently showed that there are binding sites for thrombin on the vWf protein (114). They also suggest that these are identical with the binding site for factor VIII on the vWf protein. No binding between dissociated factor VIII and thrombin has been demonstrated so far.

The effects of factor Xa, factor IXa and protein C on factor VIII

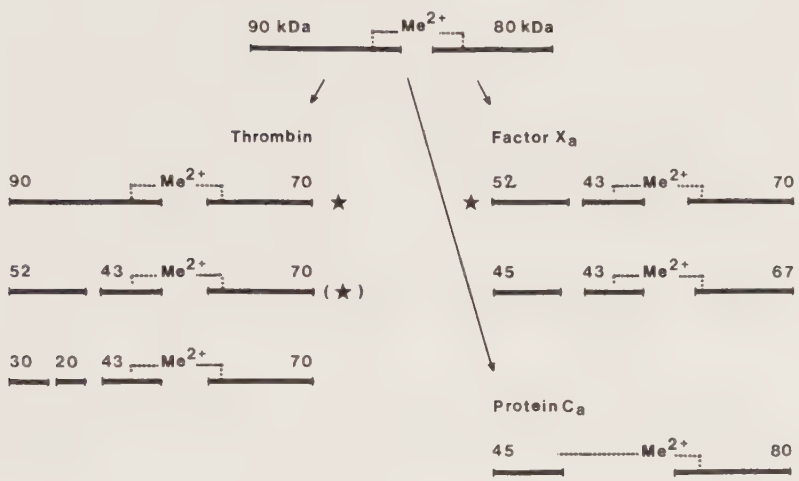
It has been established that activation of factor VIII is essential for optimal activation of factor X by factor IXa. An alternative to the thrombin activation is the activation of factor VIII by factor Xa (55) (108) (110). In this system Ca^{2+} ions and phospholipid are required, and about the same extent of activation was noted as when thrombin was used. The proteolytic cleavage of the heavy chain, resulted in the formation of a 90 kDa peptide chain which was further degraded and a 52 kDa, a 45 kDa and a 43 kDa chain was isolated from this portion of the heavy chain. The cleavage of the light chain of 80 kDa was the same when factor Xa was used as activator as was seen after treatment with thrombin (108). The 70 kDa peptide chain formed was further cleaved into a 67 kDa peptide chain. These final products of the action of factor Xa on factor VIII were suggested to correlate with the inactivation of the factor VIIIa formed (see fig. 8).

Vehar and Davie found that the action of factor Xa and thrombin on factor VIII results in an increase in its activity, whereas factor IXa does not activate factor VIII (55). This indicates that different mechanisms are involved in the effect of factor Xa or thrombin and the effect of factor IXa on factor VIII. However, Rick reported a 4-5-fold activation of factor VIII by factor IXa when a considerably larger amount of factor IXa was used (111).

The combination of factor IXa and phospholipid was shown by Lollar et al. to preserve the enhanced factor VIII activity produced by incubation with thrombin in a system using porcine protein components (112). There was no effect on the decrease in activity when factor IXa or phospholipid was used independently. However, it has been shown in our laboratory that an addition of phospholipid before or during the activation of human factor VIII-vWf stabilizes the activity and the extent of activation by thrombin is increased (113). The discrepancy in these results may be explained by the variation in species used and in the purity of material, but also by the variation in the methodology employed. In the study by Lollar et al. a ratio of 1:33 or 1:333 (IU factor VIII/NIH U thrombin) was used, whereas our study was made with trace amounts of thrombin, the ratio being 1:0.001 (IU factor VIII/NIH U thrombin).

Protein C is the precursor of a serine protease which plays an important role in the inhibition of blood clotting (116). The activated form of protein C has been shown to be a potent inhibitor of factor VIII activity (110) (117). The inactivation was related with proteolytic cleavages in the heavy chain of factor VIII and a peptide chain of 45 kDa was formed as the high molecular weight peptides of the heavy chain (200-90 kDa) disappeared. The factor VIII molecule was shown by Bertina et al. to be protected against the action of protein Ca if factor IXa was present (117). They suggested that factor IXa and protein Ca compete for the same portion of the factor VIII molecule.

The proteolytic cleavages of factor VIII by thrombin, factor Xa and protein Ca are shown in fig. 8.



★ active form of factor VIII.

Fig. 8. Proteolytic cleavages of factor VIII by thrombin, factor Xa and protein Ca.

GENERAL SUMMARY AND CONCLUSIONS

The results of this investigation show that:

- Factor VIII circulates as a complex with the von Willebrand factor protein. This association is an equilibrium, in which 10-20% of factor VIII is probably in a free non-complexed form.
- The factor VIII-von Willebrand complex can be dissociated by adding Ca^{2+} ions. This dissociation starts at 20 mM CaCl_2 and is completed at 0.2 M CaCl_2 . However, the binding is not mediated by Ca^{2+} ions, since it occurs in the presence of chelating agents.
- Human factor VIII has a MW of 280 kDa and circulates as a two-chain protein with MW's of 200 and 80 kDa, respectively.
- The heavy and light chains of factor VIII are held together by one or several metal ion bridges. The presence of these metal ions is essential to the biological activity of factor VIII.
- Factor VIII is highly susceptible to proteolytic degradation and the heavy chain is degraded to peptides with a MW of 185-90 kDa. These fragments of the heavy chain form complexes with the light chain. The smallest active and stable form of factor VIII is composed of one 90 kDa and one 80 kDa peptide chain.
- The procoagulant activity of factor VIII is stabilized by the presence of phospholipids. Factor VIII binds to the surface of released platelets or to phospholipid vesicles containing negatively charged phospholipids. The binding to phospholipid causes a dissociation of factor VIII from the vWf protein. The degree of binding was enhanced when thrombin-activated factor VIII was used.

- The apparent antigenic concentration of factor VIII was decreased when bound to phospholipid.
- Thrombin has both a positive and negative feed-back effect on factor VIII activity. The factor VIII activity was increased by incubation with trace amounts of thrombin. The degree and rate of activation was dependent on the concentration of thrombin. A proteolytic degradation was related to the increase in factor VIII activity. The combination of one 90 kDa and one 70 kDa peptide chain represents one active form of factor VIII.
- The activation of factor VIII is followed by a decrease in activity. This inactivation is probably due to a conformational change in the factor VIII molecule since the loss of activity continued although the peptide composition was preserved by removal or inhibition of the thrombin activity.

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